

DR. RAUL CARLOS MAINAR-JAIME (Orcid ID : 0000-0001-5442-7702)

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Corresponding Author Email ID: rcmainar@unizar.es

Wild griffon vultures (*Gyps fulvus*) fed at supplementary feeding stations: potential carriers of pig pathogens and pig-derived antimicrobial resistance?

Running title: Pig pathogens in wild griffon vultures

Eloisa Sevilla¹, Clara Marín², José F. Delgado-Blas³, Bruno González-Zorn³, Santiago Vega², Ed Kuijper⁴, Rosa Bolea^{1#}, Raúl C. Mainar-Jaime^{1*#}

¹Dpto. de Patología Animal, Facultad de Veterinaria, Instituto Agroalimentario de Aragón - IA2 - (Universidad de Zaragoza-CITA), Zaragoza, Spain; ²Instituto de Ciencias Biomédicas, Universidad CEU Cardenal Herrera, CEU Universities, Valencia, Spain; ³Dpto. de Sanidad Animal y Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Facultad de Veterinaria, Universidad Complutense de Madrid, Spain; ⁴Dpt. of Medical Microbiology, Centre of Infectious Diseases, Leiden University Medical Centre, Leiden, The Netherlands.

#Rosa Bolea and Raúl C. Mainar-Jaime are equal senior contributors to this work.

*Corresponding author.

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SUMMARY

The carriage of two important pathogens of pigs, i.e. enterotoxigenic *E. coli* (ETEC) and *Clostridioides difficile*, was investigated in 104 cloacal samples from wild griffon vultures (*Gyps fulvus*) fed on pig carcasses at supplementary feeding stations (SFS), along with their level of antimicrobial resistance (AMR). *E. coli* was isolated from 90 (86.5%) samples but no ETEC was detected, likely because ETEC fimbriae confer the species specificity of the pathogen. Resistance to at least one antimicrobial agent was detected in 89.9% of *E. coli* isolates, being AMR levels extremely high (>70%) for tetracycline and streptomycin, and very high (>50%) for ampicillin and sulfamethoxazole-trimethoprim. Resistance to other critically important antimicrobials such as colistin and extended-spectrum cephalosporins was 2.2%, and 1.1%, respectively, and was encoded by the *mcr-1* and *bla*_{SHV-12} genes. Multidrug resistance was displayed by 80% of the resistant *E. coli* and *bla*_{SHV-12} gene shared plasmid with other AMR genes. In general, resistance patterns in *E. coli* from vultures mirrored those found in pigs. *C. difficile* was detected in three samples (2.9%), two of them belonged to PCR-ribotype 078 and one to PCR-ribotype 126, both commonly found in pigs. All *C. difficile* isolates were characterized by a moderate to high level of resistance to fluoroquinolones and macrolides but susceptible to metronidazole or vancomycin, similar to what is usually found in *C. difficile* isolates from pigs. Thus, vultures may contribute somewhat to the environmental dissemination of some pig pathogens through their acquisition from pig carcasses and, more importantly, of AMR for antibiotics of critical importance for humans. However, the role of vultures would likely be much lesser than that of disposing pig carcasses at the SFS. The monitoring of AMR, and particularly of colistin resistant and ESBL-producing *E. coli*, should be considered in pig farms used as sources of carcasses for SFS.

Keywords: *Clostridium difficile*, Drug resistance, *Escherichia coli*, Virulence factors, Vultures

INTRODUCTION

Spain holds more than 95% of the European population of griffon vultures (*Gyps fulvus*) (Green, Donázar, Sánchez-Zapata, & Margalida, 2016), with an estimate of more than 50,000 individuals in the last official census in 2008 (Del Moral, 2009). This wild bird species keeps a particular and close association with domestic animal and, in particular, with pigs, because they may feed on pig carcasses disposed in supplementary feeding stations (SFS) available all over the country as a tool for vulture conservation (Cortés-Avizanda et al., 2016). Pig carcasses are the primary source of food at these SFS because of the vast surplus of dead pigs available (Marin et al., 2018). Indeed, Spain currently holds 30 million pig heads,

which accounts for 20% of the total European pig population, being therefore the country with the largest pig census in the European Union (Anonymous, 2018).

This feeding management, common in most regions of Spain (Del Moral, 2009), may favor the potential transmission of pathogens from animal carcasses to vultures. It is well documented that griffon vultures can act as carriers of zoonotic pathogens commonly found in pigs, such as *Salmonella* or *Campylobacter*, although their susceptibility to infection seems to vary depending upon the pathogen under consideration. For instance, recent studies have shown a high genetic similarity between *Salmonella* strains isolated in pig farms and those isolated in vultures fed on pig carcasses provided at these SFS, suggesting the potential role of SFS for dissemination of *Salmonella* to wild griffons (Blanco, 2018; Marin et al., 2018). However, this relationship was not found for *Campylobacter* (Marin, Palomeque, Marco-Jiménez, & Vega, 2014).

Less investigated has been the dissemination through SFS of other potential pathogens that affect livestock, such as enterotoxigenic *E. coli* (ETEC) and *Clostridioides difficile*. ETEC is an important cause of neonatal and post-weaning diarrhea in pigs, leading to significant economic losses. It is characterized by the presence of several virulence factors among which the most commonly identified are the fimbria F4 (K88), the heat-stable toxin a (STa), the heat-stable toxin b (STb) and the heat-labile toxin (LT) (Luppi et al., 2016). ETEC has been already found in wild birds (Kullas, Coles, Rhyan, & Clark, 2002; Silva, Nicoli, Nascimento, & Diniz, 2009), but the role that birds, and especially vultures fed at SFS, could play in the spread and maintenance of porcine ETEC remains still unknown.

C. difficile, besides of being involved in piglet neonatal diarrhea, is the leading cause of nosocomial infective diarrhea in humans, usually associated with a course of antibiotic treatment, and is now considered an emerging community-acquired infection (Smits, Lyras, Lacy, Wilcox, & Kuijper, 2016). This spore-forming pathogen has been isolated from the environment and diverse animal species, including food-producing animals (mainly pigs, cattle and poultry) and their products (Warriner, Xu, Habash, Sultan, & Weese, 2017). Due to the observed epidemiological changes and the high genetic similarity between human and animal isolates, a zoonotic transmission of *C. difficile* has been proposed (Knetsch et al., 2018). Therefore, the impact that wild birds in close contact with dead livestock at SFS could have on the carriage and dissemination of *C. difficile* also needs to be assessed.

Vultures can also act as an environmental reservoir of resistant bacteria (Blanco, 2018; Sharma, Maherchandani, Shringi, Kashyap, & Sundar, 2018), since most of the pathogens that can be acquired from pig carcasses are usually associated with high levels of antimicrobial resistance (AMR) (Sanchez-Maldonado et al., 2017). Of particular interest is the potential dissemination in the environment of

resistance to critically important antimicrobials for humans (e.g. extended-spectrum cephalosporins or colistin) (WHO, 2019), already present in bacteria isolated from pigs (Carattoli et al., 2017).

The class of beta-lactam antibiotics has become one of the most used in pig production against Gram-negative bacteria (Van Rennings et al., 2015), especially for the treatment of enteritis and respiratory, septicemia, polyarthrititis and polyserositis infections (Seiffert et al., 2013). The consumption of penicillins and cephalosporins in veterinary medicine in Spain from 2011 to 2016 was estimated as an average of 80.9 mg/PCU and 0.35 mg/PCU, respectively. With regard to their relative use among the main food-producing animal species, more than 58% of the amount of penicillins and 40% of that of cephalosporins were used in pigs in 2016 (AEMPS, 2018). The occurrence of extended-spectrum beta-lactamase-producing *E. coli* (ESBL-*E. coli*) is of major concern due to the fact that this type of resistance is mostly mediated by a wide range of mobile genetic elements that are selected through the use of these type of antibiotics (Paterson & Bonomo, 2005). In fact, the spread of ESBL-*E. coli* in the environment and wild animal populations has been already detected, being the majority of the sequence types found in isolates from wildlife origin also present in isolates from humans and domestic animals (Guenther, Ewers, & Wieler, 2011).

Colistin, a last-resort antibiotic used in human medicine for the treatment of multidrug resistant Gram-negative bacterial infections, has been widely used to treat infections caused by *Enterobacteriaceae* in food-producing animals, mainly swine and poultry (EMA, 2016). The emergence and spread of transferable plasmid-mediated colistin resistance (*mcr*) genes, found in bacteria from human, animal and environmental origin, has resulted in an alarming health threat (Sun, Zhang, Liu, & Feng, 2018). Wildlife isolates harboring *mcr-1*, while less documented, have been already reported, with six of them being isolated from gulls, one from a swallow, one from a common coot, and another one from a macaque (Dolejska & Papagiannitsis, 2018). The spread of AMR, and especially resistance to this type of antimicrobials, may pose a serious therapeutic challenge for public and animal health, particularly in Spain, a country that has the highest sales of antimicrobials for veterinary use in Europe (EMA, 2018).

Thus, the main aim of this study was to investigate the carriage of two important pathogens of pigs (ETEC and *C. difficile*) in griffon vultures fed on swine carcasses at SFS. In addition, the level of AMR was also assessed through the analysis of indicator *E. coli* isolates from these vultures.

MATERIALS & METHODS

-Population of study

The study was conducted within the conservation project for endangered species in the Valencia Region. A total of 104 cloacal samples were collected in two sessions in October 2016 through sterile cotton swabs (Cary Blair sterile transport swabs, Deltalab, Barcelona, Spain) from wild griffon vultures (*Gyps fulvus*). The study population was located at the Cinctores observatory (Castellón province, eastern Spain). In 2008, 236 breeding pairs were found in this area (93% of the breeding pairs in the Community of Valencia) (GVA, 2008). This population of vultures received supplementary feeding consisting of pig carcasses from 11 intensive-rearing farms authorized to provide carcasses to the SFS point within the reserve.

The sampling took place during the normal ringing schedule of the observatory, as part of the reserve's monitoring program. Animals were live-captured using a remotely activated purpose-built cage, and classified as juvenile (<2 years), sub-adult (from 2 to 5 years) and adult (>5 years) according to the plumage characteristics and the bill and eye color.

All the experimental procedures used in this study were performed in accordance with Directive 2010/63/EU EEC on animal experiments. The Department of Infrastructure, Planning and Environment of the Valencian Regional Government (Generalitat Valenciana) granted the ethical and animal welfare permission to take samples.

In order to assess the potential presence of *C. difficile* in the environment associated with the pig carcasses, given that its spores can easily persist (Smits et al., 2016), a convenience sample of 20 environmental samples were collected using swabs from containers and trucks (walls and floors) used for the storage and transport of the carcasses, respectively, as well as from some carcasses (surface). These samples were collected at the same time as cloacal sampling.

A more detailed description on the capture of the animals and sample collection can be found elsewhere (Marín, Palomeque, Marco-Jiménez, & Vega, 2014; Marín et al., 2018).

-Isolation of ETEC

Fecal samples were pre-enriched in Buffered Peptone Water (Panreac, Barcelona, Spain) at 37°C for 24h. The pre-enriched samples were then plated onto MacConkey agar (Panreac) and incubated at 37°C for 24h. Three colonies per sample resembling *E. coli* were then selected and screened for indole production. Those colonies confirmed as *E. coli* were stored at -30°C until further analysis.

For detection of virulence factors associated with porcine ETEC, i.e. the fimbrial adhesin F4 (K88), heat stable enterotoxins (STa and STb) and heat labile enterotoxin (LT), DNA was first extracted from pure

culture by boiling. To evaluate the presence of genes encoding the above mentioned virulence factors, multiplex Polymerase Chain Reaction (PCR) was performed using a procedure previously described by the Reference Laboratory for *Escherichia coli* (EcL) at the Faculty of Veterinary Medicine from the Université de Montréal (available at <http://www.apzec.ca/en/Protocols>). The strain ECL8559 (O149: STa: STb: LT: F4: Paa: EAST1) kindly provided by Dr. J.M. Fairbrother (Université de Montréal) was used as a positive control in this assay.

-Antimicrobial susceptibility testing in *E. coli* isolates

One *E. coli* isolate from each positive sample was tested for AMR against a total of 22 antibiotics included within 15 different antimicrobial classes. The selection of these drugs was based on the frequent use in clinical practice, i.e., amikacin, amoxicillin-clavulanic acid, ampicillin, ceftiofur, cephalexin, cephalothin, cefoxitin, chloramphenicol, enrofloxacin, florphenicol, fosfomycin, gentamicin, neomycin, nitrofurantoin, streptomycin, tetracycline and sulfamethoxazole-trimethoprim; and on its relevance in human medicine, i.e. ceftriaxone, ciprofloxacin, colistin, imipenem and tigecycline.

The antimicrobial susceptibility testing was performed by the Kirby-Bauer disk diffusion method, except for colistin. *E. coli* ATCC 25922 was used as a reference strain. Each isolate was categorized as susceptible, intermediate or resistant following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2017). Isolates with intermediate susceptibility results were categorized as resistant for statistical analysis.

In the case of colistin, the broth microdilution method (ISO 20776-1:2006) and epidemiological cut-off (ECOFF) value of >2 mg/L were applied, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019). Those isolates considered phenotypically resistant, that is, showing a minimum inhibitory concentration (MIC) >2 mg/L, were further studied for the presence of plasmid-mediated colistin resistance genes (*mcr*), as well as for chromosomal mutations in the PmrAB system associated to resistance to this antimicrobial agent. For this purpose, DNA was extracted from pure culture by boiling and the presence of *mcr* genes (*mcr-1* to *mcr-4*) was detected by conventional PCR as previously described (Carattoli et al., 2017; Liu et al., 2016; Xavier, Lammens, Ruhel, et al., 2016; Yin et al., 2017). Polymorphisms of *pmrA* and *pmrB* genes were screened by PCR amplification and Sanger sequencing (GenBank accession numbers MK506814 to MK506817). The results obtained were compared to pmrAB operon sequences from colistin resistant and susceptible *E. coli* described by Quesada et al. (2015). Control strains were kindly provided by Dr. B. González-Zorn, University Complutense of Madrid, Spain (*mcr-1*), Dr. R. S. Hendriksen, Technical University of Denmark (*mcr-2*), Dr. Y. Wang, China Agricultural University (*mcr-3*), and Dr. A. Carattoli, Istituto Superiore di Sanità, Italy (*mcr-4*).

The measurement of the inhibition zone corresponding to ceftriaxone, a third-generation cephalosporin, was used as a screening test for the production of extended-spectrum β -lactamases (ESBLs) (CLSI, 2017). Thus, those *E. coli* isolates displaying reduced susceptibility to this agent were subjected to a confirmatory test through the Total ESBL + AmpC Confirm Kit (Rosco Diagnostica, Taastrup Denmark). This kit consists of 6 discs containing cefotaxime and ceftazidime alone or combined with β -lactamase inhibitors (i.e. clavulanate and/or cloxacillin). As defined by manufacturer's instructions, if a difference of ≥ 5 mm was observed between the inhibition zones of the discs containing a cephalosporin (i.e. cefotaxime or ceftazidime) plus cloxacillin with and without clavulanate, the tested isolate was considered ESBL positive. If a difference of ≥ 5 mm was detected between the inhibition zones of the discs containing a cephalosporin (i.e. cefotaxime or ceftazidime) plus clavulanate with and without cloxacillin, the tested isolate possessed AmpC.

Overall levels of antimicrobial resistance were defined according to the classification proposed by the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) (2013) (Table 1). *E. coli* isolates displaying resistance to at least one agent in three or more antimicrobial classes were considered multidrug resistant (Magiorakos et al., 2012).

Further whole genome sequencing (WGS) of ESBL-producing *E. coli* was assessed by MinION sequencer (Oxford Nanopore Technologies, Oxford, UK). DNA genomic extraction was performed with Wizard Genomic DNA Purification kit (Promega, Madison, USA) and DNA quality and concentration were measured by NanoDrop (Thermo Fisher Scientific, Wilmington, USA) and Qubit (Invitrogen, Carlsbad, USA) devices. Genomic library was performed following the 1D Native barcoding genomic DNA protocol, with EXP-NBD104 and SQK-LSK109 kits (Oxford Nanopore Technologies), and sequencing was run in a FLO-MIN106 flow cell. Downstream analysis was performed as follows: sequencing reads were basecalled with MinKNOW software (Oxford Nanopore Technologies), demultiplexing process was carried out with the barcoding pipeline of Epi2Me interface (Metrichor, Oxford, UK) and trimming of adaptors and barcodes from the reads was assessed by Porechop. Long reads assembly was achieved by Canu (Koren et al., 2017), and subsequent genomic assemblies were analyzed with Bandage (Wick, Schultz, Zobel, & Holt, 2015) and BLAST+ (Camacho et al., 2009), including the Resfinder and Plasmidfinder databases, in order to determine the total resistance gene and plasmid content, respectively, and the location of these genes in the genome.

-Isolation of *C. difficile*

Detection of *C. difficile* was carried out in accordance with the protocol described elsewhere (Andrés-Lasheras et al., 2017). Briefly, cloacal and environmental samples were pre-enriched in 9 mL of Brain Heart Infusion (Oxoid, Basingstoke, Hampshire, UK) supplemented with *C. difficile* selective supplement (C.D.M.N. Selective supplement; Oxoid), 6 g/L fructose (Sigma-Aldrich, St. Louis, Missouri, US) and 1 g/L sodium taurocholate (Sigma-Aldrich), and incubated anaerobically at 37°C for six days. After this, 2 mL of pre-enriched broth was mixed 1:1 vol/vol with ethanol 96°, and incubated at room temperature for 1h. Samples were then centrifuged at 4000 rpm for 15 min. The supernatants were discarded and the precipitates were plated on cycloserine-cefoxitin agar (CLO agar; bioMérieux, Marcy l'Etoile, France). Plates were incubated under anaerobic conditions at 37°C for 48h, and for a further 72 h when no *C. difficile* growth was observed. Bacteriological identification was based on colony morphology, typical odor (horse manure) and Gram stain. Three colonies per positive sample were isolated and stored at -30°C until further analysis.

Confirmation of *C. difficile* isolates was performed by amplification of the specific glutamase dehydrogenase (*gluD*) gene (Paltansing et al., 2007). Toxin A (*tcdA*) and B (*tcdB*) and the binary toxin (*cdtA / cdtB*) genes were also detected by PCR (Persson, Torpdahl, & Olsen, 2008). Capillary ribotyping was carried out according to the Leeds-Leiden database (Fawley et al., 2015).

Additionally, *C. difficile* isolates were tested for antimicrobial resistance against seven agents, including ciprofloxacin, clindamycin, erythromycin, metronidazole, moxifloxacin, vancomycin and tetracycline. The antimicrobial susceptibility testing was performed by Etest (Liofilchem, Teramo, Italia) on *Brucella* blood agar supplemented with hemin and vitamin K1 (Sigma-Aldrich). Plates were incubated anaerobically at 37°C for 48h, and for up to five days in order to detect slow-growing metronidazole-resistant subpopulations (T. Peláez et al., 2008). Breakpoints used for antimicrobial resistance categorization were those established by CLSI (2017) or, if not available as in the case of ciprofloxacin, erythromycin and vancomycin, based on the literature (Álvarez-Pérez et al., 2014).

-Statistical analysis

Prevalence estimates with their 95% Confidence Intervals (95% CI) were calculated. When required, comparisons for prevalence of ETEC and *C. difficile* among age categories were made using the Fisher-exact test, as some cell values were below five. A difference was considered statistically significant for a *P*-value ≤ 0.05 . All the analyses were performed using MedCalc v. 18.10 (MedCalc, Ostend, Belgium).

RESULTS

From the 104 wild griffon vultures sampled 9 (8.7%) were juveniles, 17 (16.3%) sub-adults and 78 (75%) adults.

-Isolation of *E. coli* and ETEC virulence factors

E. coli was isolated from 90 (86.5%; 95% CI: 78.7-91.8) out of the 104 cloacal samples, but no differences in the percentage of recovery of *E. coli* was observed among age categories (88.8% in juveniles, 100% in sub-adults, and 83.3% in adults; $P=0.18$). None of the ETEC virulence factors investigated (i.e. F4, STa, STb and LT) were detected in these isolates.

-Antimicrobial susceptibility testing in *E. coli* isolates

The proportion of *E. coli* isolates resistant to each of the antibiotics tested and the general level of resistance for each of them are shown in Table 1. Resistance to at least one antimicrobial agent was detected in 89.9% (95% CI: 82.1-94.6) of the isolates. AMR levels were extremely high (>70%) for tetracycline and streptomycin, and very high (>50%) for ampicillin and sulfamethoxazole-trimethoprim. With regard to the critically important antimicrobials for human medicine (WHO, 2019), the AMR level observed was from rare (imipenem, amikacin, and fosfomycin) to extremely high (streptomycin). Interestingly, resistance to colistin, considered a last-resort antimicrobial, was present in 2.2% of the isolates (Table 1). Multidrug resistance (MDR) was also extremely high with 80.2% (95% CI: 70-87.5) of all resistant *E. coli* isolates displaying phenotypic resistance to three or more antimicrobial classes.

Two (2.22%; 95% CI: 0.61-7.74) *E. coli* strains showed phenotypic resistance to colistin (MIC = 4 mg/L). The polymorphisms observed in the *pmrA* and *pmrB* coding sequences for these two strains were either synonymous or non-synonymous but not unique, i.e. occurring in other colistin-susceptible *E. coli* sequences. However, both isolates encoded the plasmid-born *mcr-1* gene. No other *mcr* genes were detected. These colistin resistant isolates also showed a MDR profile, including resistance to aminoglycoside, fluoroquinolone, phenicol, sulfonamide and pyrimidine and tetracycline classes.

One (1.1%; 95% CI: 0.2-6.03) *E. coli* isolate showed reduced susceptibility to ceftriaxone, and the presence of ESBL production was confirmed afterwards. This isolate also showed a MDR profile (i.e. penicillins - aminoglycosides - phenicols - sulfonamide and pyrimidine - fluoroquinolones). The WGS analysis identified the presence of the *bla_{SHV-12}* gene, which is associated with ESBL production, along with *aadA1*, *aadA2*, *aph(3'')-Ib*, encoding for resistance to aminoglycosides, *mdf(A)* encoding for resistance to macrolides, *cmlA1* for phenicols, and *sul3* for sulfonamides. The *bla_{SHV-12}*, *aadA1*, *aadA2*, *cmlA1* and *sul3* were associated to a single IncI1 plasmid, while the *aph(3'')-Ib* gene was associated to a IncFII plasmid, and the *mdf(A)* gene was located within the chromosome.

No statistically significant differences percentage of AMR for the different antibiotic classes were found among age categories, except for phenicols (Figure 1). The prevalence of MDR among juvenile, sub-adult and adult individuals was not significant as well (65.5%, 70.6% and 72.3%, respectively; $P=0.84$).

-Isolation of *C. difficile*

C. difficile was isolated in three cloacal samples (2.9%; 95% CI: 0.9-8.1). Two isolates came from adult vultures and one from a juvenile (Table 2). No evidence of *C. difficile* growth was observed in the bacteriological culture of any of the environmental samples.

All isolates were positive for the toxin genes *tcdA*, *tcdB*, *cdtA* and *cdtB*, belonging two of them to PCR-ribotype 078 and one to PCR-ribotype 126. According to the antimicrobial susceptibility testing, all *C. difficile* isolates were considered resistant to ciprofloxacin, two (66.6%) to moxifloxacin and one (33.3%) to erythromycin. Additionally, intermediate resistance was observed for clindamycin (66.6%) and tetracycline (33.3%). No resistance to metronidazole or vancomycin was detected in these *C. difficile* isolates.

DISCUSSION

Scavengers are considered potential environmental indicators of the presence of zoonotic pathogens due to their eating habits as they prey on animal carcasses and rubbish (Plaza et al., 2019). In Spain, apart from feeding on wild ungulates carcasses, vultures used to forage on dead livestock (mostly ruminants) left by farmers on the field or at SFS. However, after the first cases of Bovine Spongiform Encephalopathy appeared two decades ago, SFS were mostly filled with pig carcasses. Today griffon vultures are almost exclusively feed on this type of food (Blanco, 2018; Camiña & Montelío, 2006). The particular and intimate relationship between vultures and dead pigs at these SFS would make these birds good indicators of the presence of some pig pathogens (zoonotic or not), as they may become infected by eating contaminated pig carcasses. Vultures could even act as carriers, helping to the spread of these pathogens, and also of their AMR. Indeed, it has been observed that vultures are carriers of virulence genes typical of extraintestinal pathogenic *E. coli* (ExPEC) (i.e. *fimH*, *fimA*, *MT78*, *iroN*, *iucD*, *cvaC*, *iss*, *traT* and *tsh*) although they do not show ExPEC status (Mora et al., 2014). Therefore, we hypothesized that they could also harbor virulence genes of ETEC acquired from pig carcasses, but none of the 90 *E. coli* strains isolated from these vultures was identified as ETEC. To detect ETEC F4-fimbriae were sought because they may be found in pigs of all ages as receptors for this type of adhesins are fully expressed from birth to adult age (Fairbrother, Nadeau, & Gyles, 2005). The F4-ETEC variant is frequently isolated from pigs among

European countries (Luppi et al., 2016), and is present in more than 20% of the pig farms in Spain (Pérez, Claver, Piqué, & Sánchez, 2016). Thus, the failure to detect ETEC in these vulture samples suggest that they are not easily colonized by this pathotype of *E. coli* and therefore vultures would present a very limited role, if any, in the spread of this pathogen. In fact, to the author's knowledge, there is just a couple of studies reporting on the presence of ETEC in wild bird species, but without describing the associated fimbriae (Kullas et al., 2002; Silva et al., 2009). Lack of colonization would be most likely due to host specificity of fimbrial adhesins, as ETEC fimbriae confer the species specificity of the pathogen, making difficult to infect other animal species or humans (Fairbrother et al., 2005).

Of special interest is the role that vultures may have on the dissemination of AMR as intensive pig production is nowadays associated with the presence of high levels of resistant bacteria (Brilhante, Perreten, & Donà, 2019; Burow et al., 2019). Some studies have shown that prevalence of AMR against aminoglycosides, penicillins, sulfamethoxazole-trimethoprim and tetracycline in bacteria from the *Enterobacteriaceae* family is common in pigs in Spain, and usually above 70% (Pérez et al., 2016; Teshager et al., 2000; Vico et al., 2011). In addition, similar resistance profiles have been observed between bacterial isolates from pigs and wild animal species captured close to the farms where these pigs were raised (Allen et al., 2011; Andrés-Barranco et al., 2014). Thus, the extremely high or very high levels of AMR found for tetracycline, streptomycin, ampicillin and sulfamethoxazole-trimethoprim in *E. coli* from these griffon vultures would likely be related to the alarming AMR scenario observed in swine. In general, no differences in prevalence of AMR/MDR were observed among age categories, suggesting that the likelihood of exposure to resistant *E. coli* strains was similar for juvenile, sub-adult and adult vultures, as all fed on the same SFS.

Particularly worrisome is the prevalence of MDR, also above 70%, and especially when is associated to AMR against antimicrobials of critical importance for human medicine such as colistin or ceftriaxone (a third-generation cephalosporin). Two (2.22%) *E. coli* isolates displayed resistance to colistin, which seemed to be linked to the presence of the plasmid-borne gene *mcr-1* and not to chromosomal mutations (polymorphisms in the *pmrA* and *pmrB* coding sequences were also present in colistin-susceptible *E. coli*). This gene would have a high capacity of spread through mechanisms of horizontal genetic transfer (Shen et al., 2018).

The *mcr-1* gene was first detected in 2015 in China (Liu et al., 2016) and since then it has been found worldwide in *Enterobacteriaceae* isolates, mostly *E. coli* of pig origin, but also from other food-producing animals (Anjum et al., 2016; Perrin-Guyomard et al., 2016; Xavier et al., 2016). The presence of the *mcr-1* gene in wildlife has been however less reported, likely due to the lower contact of wildlife with polimixins.

mcr-1 has been detected in *E. coli* strains isolated from European herring gull (*Larus argentatus*) and Kelp gulls (*Larus dominicanus*) feces in Lithuania and Argentina, respectively (Liakopoulos, Mevius, Olsen, & Bonnedahl, 2016; Ruzauskas & Vaskeviciute, 2016). Gulls usually feed on organic waste from rubbish dumps where the likelihood of getting in contact with pathogens, mostly from the *Enterobacteriaceae* family, is much higher (Plaza et al., 2019). Thus, it appears that scavengers will be at higher risk of harboring *mcr-1*-positive *E. coli*, and therefore may contribute to its environmental dissemination.

Since colistin has been widely used for treating porcine neonatal and post-weaning diarrhea (EMA, 2016), there might be a link between resistance to this antimicrobial and pathogenic *E. coli* strains in pigs. A recent study suggests that the prevalence of *mcr-1* gene in *E. coli* from diarrheagenic neonatal and post-weaned pigs in Spain must be high, as this gene was found in 25.6% of the diarrheagenic *E. coli* isolates collected between 2006 and 2016 from pigs suffering of enteric colibacillosis in different Spanish regions (García-Meniño et al., 2018). Therefore, the presence of pig carcasses contaminated with *E. coli* harboring the *mcr-1* gene at these SFS should be expected. The lower prevalence of *mcr-1* detected in vultures may be likely associated to the fact that no ETEC were isolated in these wild birds. Nevertheless, commensal *E. coli* are regarded as good indicators of AMR and could thus be reservoir of this type of genes for vultures.

Both colistin-resistant *E. coli* isolates displayed resistance to multiple antimicrobial classes (penicillins - non-extended spectrum cephalosporins - aminoglycosides - phenicols - tetracyclines - sulfonamide and pyrimidine - nitrofurans; and aminoglycosides - phenicols - tetracyclines - sulfonamide and pyrimidine - fluoroquinolones). The possible co-existence of multiple resistance genes on the same plasmid may facilitate the dissemination of colistin resistant strains by the co-selective pressure applied by the presence of other antibiotics (Dandachi, Chabou, Daoud, & Rolain, 2018). Considering that pig carcasses usually belong to sick animals that could have been medicated with antibiotics just before dying, it can be hypothesized that the presence of antibiotic residues in these carcasses could contribute to selective pressure in the gut microbiota of vultures and lead to co-resistance to colistin. Indeed, the presence of residues of fluoroquinolones in the plasma of vultures has been already observed, likely coming from medicated livestock carcasses (Blanco et al., 2016). Further studies on the presence of antibiotic residues on meat from pig carcasses at SFS would help to clarify this issue.

Regarding ESBL-producing *E. coli*, it has been frequently detected in wild bird species worldwide, including Spain (see review in Wang et al. 2017). Apart from pig carcasses, no other major sources of ESBLs were expected for vultures, since, outside of SFS, they feed mostly on carcasses from wild ungulates, which are not expected to have been exposed to antibiotics. The prevalence of ESBL-producing *E. coli* in griffon vultures has been less studied and usually the studies have been performed on a low

number of *E. coli* strains. Two studies in Spain analyzed *E. coli* isolates collected in 2011 (Mora et al., 2014) and 2013-2014 (Alcalá et al., 2016) and showed null (0 out of 14) and high (21.4%; 3 out of 14) prevalence of ESBL-producing *E. coli*, respectively. The most recent study of this type in Spain, on 91 *E. coli* isolates, reported up to 26% of ESBL producers (Lopez Cerero, López-Hernández, Blanco, & Javier, 2017). We analyzed a similar number of *E. coli* isolates (90) and during the same year (2016), and our results however suggested that the prevalence of ESBL-producing *E. coli* would be much lower (1.1%). The discrepancies observed in prevalence of ESBL-producing *E. coli* among studies may be associated with many factors. One of these would be the different years in which the studies were performed, which could suggest an overall increase of this prevalence (from 2011 to 2016) in these wild species, mirroring what was occurring for pigs (Cortés et al., 2010; Van Damme et al., 2017). However, some bias should be also expected due to both the small sample size used in the first two studies and the probable clustering of the sampled vultures (i.e. within a given SFS and geographical area). Indeed, geographical differences have been observed in the occurrence of this type of resistance (Wang et al., 2017), and are more likely responsible of these differences. For instance, this study and Mora's were carried out on vultures from areas of eastern Spain characterized by a moderate-low pig production, while the vultures from the other two studies showing high ESBL-producing *E. coli* prevalences were located in important pig-producing areas of central and northeast Spain. Thus, the likelihood that vultures were exposed to more carcasses from antibiotic-treated pigs would be likely higher in the latter. The type of pig production (intensive vs. outdoor) was not considered a factor of variability as all the mentioned studies were carried out in intensive pig-production areas.

The CTXM-type β -lactamases are the most common ESBLs among *Enterobacteriaceae* isolates of wild animals (Wang et al., 2017). However, the ESBL-producing *E. coli* detected in this study contained the *bla*_{SHV-12} gene. This gene was also detected in *E. coli* isolates from other Spanish vultures (Alcalá et al., 2016) and is, along with the *bla*_{CTX-M-group-9}, *bla*_{CTX-M-group-1}, and *bla*_{SHV-5}, the most detected gene in *E. coli* from pigs in Spain (Dandachi et al., 2018). Thus, this finding suggested a pig carcass-vulture transmission. This isolate was further submitted to WGS analysis and it was found that it harbored resistance genes to other antibiotic classes, i.e. aminoglycosides (*aadA1* and *aadA2*), phenicols (*cmlA1*) and sulfonamides (*sul3*) that shared the same IncI1 plasmid. This plasmid type is among the first for transmission of ESBL and AmpC-encoding genes in isolates from food-producing animals (Carattoli, Villa, Fortini, & García-Fernández, 2018). As for colistin, the presence of several genes encoding for other types of AMR within the same plasmid would imply a higher risk of maintenance of resistance to 3rd generation cephalosporins even when these type of antibiotics are not present in the pig carcasses.

Regarding *C. difficile*, although the prevalence of carriage is usually null or low in wild birds (Bandelj et al., 2014, 2011), it is detected in pest species (or their feces) present in the surroundings of pig farms (Andrés-Lasheras et al., 2017; Burt, Siemeling, Kuijper, & Lipman, 2012). Thus, no species specificity was expected in this case. Three per cent of the griffon vultures harbored *C. difficile* in their feces, and to the authors' knowledge this is the first report on the isolation of *C. difficile* in wild vultures. This pathogen is quite prevalent in pigs, especially during the lactation period (Alvarez-Perez et al., 2009; Pirs, Ocepek, & Rupnik, 2008), after which prevalence of infection decreases significantly, being detectable in low levels (0.3%) in fattening pigs (Susick, Putnam, Bermudez, & Thakur, 2012). Since pig carcasses available at the SFS were mostly from fattening pigs, the prevalence of *C. difficile* in these vultures may reflect somewhat the likely low level of carcass contamination with this pathogen, which was also supported by the absence of *C. difficile* in the environmental samples analyzed. Two out of three of the *C. difficile* isolates described in this study belonged to the hypervirulent PCR-ribotype 078, the most prevalent ribotype found in porcine strains (Teresa Peláez et al., 2013; Stein et al., 2017), and the third isolate corresponded to PCR-ribotype 126, which is genetically related to ribotype 078, and also frequently found in pigs (Schneeberg et al., 2013). The antimicrobial susceptibility profile of these vulture isolates, characterized by a moderate to high level of resistance to fluoroquinolones and macrolides, was also similar to that found in *C. difficile* isolates from porcine origin (Keessen et al., 2013; Teresa Peláez et al., 2013; Spigaglia et al., 2015). No resistance to metronidazole or vancomycin, two first-line antimicrobials currently used for the treatment of *C. difficile* infection in humans, was observed in this study, which is also rare among porcine isolates (Teresa Peláez et al., 2013; Spigaglia et al., 2015; Zhang et al., 2019). All together supported the more than likely transmission of this pathogen from pig carcasses to vultures. Since it is the first time that *C. difficile* is isolated from apparently healthy wild vultures, additional studies will be required to determine the role that this bacterium may have on vultures' health, focusing especially on digestive problems.

CONCLUSIONS

Griffon vultures may harbor pathogens of human and veterinary importance because of their eating habits. This study shows that they can become infected with *C. difficile*, but it appeared that particular pig pathotypes of *E. coli* (i.e. ETEC) may not infect these wild birds, likely due to their host specificity. A high proportion of *E. coli* isolates displaying AMR was also found, suggesting that vultures could contribute to some extent to the environmental dissemination of AMR as a likely consequence of being fed on dead pigs from commercial farms. Of major concern was the detection of *E. coli* isolates resistant to antimicrobials of critical importance for humans (colistin or extended-spectrum cephalosporins) which were also displaying a MDR profile. Considering that in Spain a major strategy for griffon vulture conservation has been the establishment of SFS as a way to increase the availability of food resources for this endangered species, these sites might be having some sort of positive impact on the spread of the pig-derived AMR. However,

the role that vultures may have on its dissemination would likely be much lesser than that of disposing pig carcasses at the SFS for consumption by them. Thus, in order to mitigate the potential spread in the environment of AMR through SFS, the monitoring of AMR, and particularly of colistin resistant and ESBL-producing *E. coli*, should be considered in those pig farms used as sources of carcasses for SFS.

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CONFLICT OF INTEREST

The authors do not have any conflict of interests.

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Table 1. Percentage of resistance among 90 *E. coli* isolates detected in fecal samples from griffon vultures (in bold antimicrobials considered as critically important for human medicine -WHO, 2019-).

Antimicrobial	% R/I	Categorization*
Tetracycline	72.22	Extremely high
Streptomycin	71.11	
Ampicillin	67.78	Very high
Sulfamethoxazole-trimethoprim	54.44	
Cephalothin	38.89	High
Nitrofurantoin	37.78	
Enrofloxacin	27.78	
Neomycin	26.67	
Chloramphenicol	25.56	
Florphenicol	11.11	Moderate
Ciprofloxacin	11.11	
Gentamicin	5.56	Low
Tigecycline	5.56	
Cephalexin	3.33	
Amoxicillin-clavulanic acid	2.22	
Colistin	2.22	
Ceftriaxone	1.11	Rare
Imipenem	0.00	
Cefoxitin	0.00	
Ceftiofur	0.00	
Amikacin	0.00	
Fosfomycin	0.00	

Intermediate (I); Resistant (R). *Rare, <0.1%; very low, 0.1–1%; low, >1–10%; moderate, >10–20%; high, >20–50%; very high, >50–70%; and extremely high, >70% (EFSA & ECDC, 2013).

Table 2. Characteristics of the three *C. difficile* isolates detected in fecal samples from griffon vultures.

<i>C. difficile</i> strain	Sample	Vulture age	PCR-ribotype	AMR
VU 31	Cloacal swab	adult	078	CIP-CD [†] -MXF
VU 33	Cloacal swab	adult	126	CIP-E
VU 139	Cloacal swab	juvenile	078	CIP-CD [†] -MXF-TE [†]

Antimicrobial resistance (AMR); Ciprofloxacin (CIP); Clindamycin (CD); Erythromycin (E); Moxifloxacin (MXF); Tetracycline (TE). [†] Intermediate resistance.

Figure 1. Percentage of resistance against different antimicrobial classes in *E. coli* isolates detected in fecal samples from griffon vultures of different age categories.

(A) Penicillins; (Ac) Penicillins+ β -lactamase inhibitors; (Ca) Carbapenems; (NSC) Non-extended spectrum cephalosporins (1st and 2nd generation); (ESC) Extended-spectrum cephalosporins (3rd generation); (Cm) Cephamycins; (S) Aminoglycosides; (C) Phenicol; (T) Tetracyclines; (G) Glycylcyclines; (Su) Sulfonamide and pyrimidine; (Na) Fluoroquinolones; (F) Phosphonic acids; (N) Nitrofurans; (P) Polymyxins; (MDR) Multidrug resistance.

Figure 1.

